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Real Time Detection of Different Variant Strains of Infectious Bronchitis Virus in Trachea, Lung and Kidney of Infected Broiler Chickens

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Abstract

BACKGROUND: Avian infectious bronchitis virus (IBV) has a great potential for genetic variability which leads to the generation of new virus strains. The changes in the IBV genome often cause alterations in virulence, tissue tropism, and viral replication in the host tissues.

OBJECTIVES: This study was conducted to identify the virus variant strains in the trachea, lungs, and kidneys of infected birds. The possible relationship of IBV variants with the relative quantity of virus in each organ was also investigated.

METHODS: The IBV variant strains were detected by polymerase chain reaction (PCR) and direct sequencing. Amongst infected commercial broiler flocks sampled at Golestan and Mazandaran provinces of Iran, nine flocks (three flocks per variant) were selected based on the identified variants. Trachea, lung, and kidney samples of five birds per flock were examined for the presence of the virus and variants. Moreover, the virus was quantified in target organs using real-time quantitative PCR.

RESULTS: Based on the results of PCR and sequencing, three IBV variants were selected, namely A, B, and C. Virus types A and B were detected in all target organs, while type C was detected in the trachea and kidney. Virus type C had the highest quantity of 17.02±5.22 and virus type A showed the lowest quantity of 5.68±2.4 in infected tissues. The relative quantity of virus detected in tissues significantly correlated with the IBV variant.

CONCLUSIONS: Genetic polymorphism in IBV field strains was revealed to have significant correlations with viral quantity in the lung, trachea, and kidney. Our findings are an update of the current knowledge on the associations between viral genotype, virulence, and pathogenicity.

KEYWORDS: Broiler, Chicken, Infectious Bronchitis Virus, Quantification, Tissue

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Introduction

Avian infectious bronchitis is one of the highly contagious viral diseases of chickens caused by a member of the genus *Gammacoronavirus* (type 3 coronavirus) named infectious bronchitis virus (IBV). It mainly affects the respiratory and renal systems of broiler chickens (Cook *et al*., 2012; de Wit and Cook, 2019) with morbidity and mortality rates of infection being closely related to the strains involved (Zhao *et al*., 2019). The virus genome contains several genes encoding structural proteins including spike (S), envelope (E), integral membrane protein (M), and nucleocapsid (N) (Boursnell *et al*., 1987). Almost two-thirds of the coronavirus genome consists of the replicase complex that is important for virus replication and codes functional domains, such as RNA-dependent RNA polymerase (RdRp), RNA helicase, papain-like protease [PL(pro)], and main protease [M(pro)] (Armesto *et al*., 2009).

The IBV is highly potential for mutation and recombination leading to the generation of new virus strains. The changes in the IBV genome often result in alterations in virulence, tissue tropism, and viral quantity in the host tissues. Most of the molecular studies have focused on the changes located at the S1 part of virus spike protein that encodes extracellular protein subdomain (Bande *et al*., 2017; Bickerton *et al*., 2018). Although S1 glycoprotein is closely related to virus antigenicity and its classic or variant serotypes (Domanska-Blicharz *et al*., 2017 & 2020), it partly determines virus virulence. The replica gene is another determinant of IBV pathogenicity because it has been demonstrated to be the only gene that can restore the virulence of an avirulent strain. Other parts of the genome, including accessory genes, are not required for virus replication in vitro and their role in pathogenicity remained unclear (Armesto *et al*., 2009).

Replicase is supposed to be a determinant of IBV pathogenicity. Therefore, variations in the RdRp gene might be associated with virus pathogenicity and tissue/cell tropism. With this background in mind, the present study aimed to determine virus variants based on the RdRp gene and investigate the possible relationships of IBV variants with the relative quantity of virus detected in the trachea, lung, and kidney.

Materials and Methods

Sampling and Study Design

Sampling, selection, and study design were based on the previously obtained data of the molecular characterization of IBV strains in the Golestan and Mazandaran provinces of Iran (Hajijafari Anaraki *et al*., 2020). The IBV variant strain in each organ was detected by polymerase chain reaction (PCR) and direct sequencing. Amongst samples collected in spring from infected commercial broiler flocks of the Golestan and Mazandaran provinces of Iran, nine flocks (three flocks per variant) were selected based on the identified variants. Trachea, lung, and kidney samples of five birds (21-28 days old) per flock were examined for the presence of viruses and variants as previously described (Hajijafari Anaraki *et al*., 2020). Furthermore, the virus was quantified in target organs using real-time quantitative PCR according to Li (Li and Handberg, 2007). Nine negative samples from non-infected birds were also examined as the control group.

RNA Isolation and Reverse Transcription PCR

Total RNA was isolated from trachea, lung, and kidney tissues using the Viral Gene-spin® Extraction Kit (Intron Biotechnology, South Korea) and was reverse transcribed to cDNA as follow: 1 µg RNA was added to 20 µL combination of 200 ng random hexamer and 0.5 mM dNTP Mix and was heated at 65°C for 5 min. After the addition of 40 unit RNase inhibitor, RT buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, and 200 unit M-MLV reverse transcriptase (Fermentas, Germany), the mixture was incubated for 10 min at 25°C followed by 50 min at 37°C. The cDNA was then heated at 75°C for 15 min and was stored at -20°C.

Conventional PCR

Two PCR primer sets were designed based on all available IBV RdRp gene sequences described earlier (Hajijafari Anaraki *et al*., 2020). Primers were specific for avian IBV and a spanning variable region of RdRp gene sequences (AY692454: nt 14167–14188 and nt 14436–14455). The PCR primers were located in polyprotein 1b. Target sequences were amplified in each sample according to our previous experiments (Hajijafari Anaraki *et al*., 2020).

Sequencing and Data Analysis

Amplified sequences were purified using the purification kit (Bioneer, Seoul, Korea) and were subjected to a direct sequencing method. All PCR products were separately sequenced in both directions by specific forward and reverse primers. Sequencing was performed by the Sanger sequencing method on an automatic DNA sequencer (ABI 3730 XL, Macrogen Inc. Seoul, Korea). Sequencing results were analyzed by BLAST through the national center for biotechnology information website (http://www.ncbi.nlm.nih.gov/). Bioedit version 7.0.5.3 software package was utilized for alignments and graphs.

Real-time Quantification of Virus

The relative viral quantity was determined by real-time PCR according to Li (Li and Handberg, 2007). The partial RdRp gene of avian IBV (288 bp) was amplified using Qiagen real-time PCR machine (Rotor-Gen Q). Similar to the conventional PCR, RdRp primers were used for the real-time quantification of the virus. In addition, 5′-GGTGGTGCTAAGCGTGTTAT-3′ and 5′-ACCTCTGTCATCTCTCCACA-3′ (K01458) primers were applied for GAPDH housekeeping gene amplification as forward and reverse primers, respectively. Each tissue sample was submitted to IBV RdRp and GAPDH cDNA real-time amplification (efficiency 96%, linearity range of Ct 18 to 35).

Amplification was performed in a final volume of 25 µL containing 20 ng template cDNA, 1.5 mM $MgCl_2$, 250 µM of each dNTP, PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1 µL (2 µM) Syto9® dye (Life Technologies Corp., Carlsbad, CA), Taq DNA polymerase (2.5 U) (CinnaGen, Tehran, Iran), and specific primers (20 pmol). The thermal cycling program was as follow 94°C for 2 min (1 cycle), followed by three steps of 95°C for 30 sec, 61°C for 30 sec, and 72°C for 40 sec (30 cycles), as well as a final extension of 10 min at 72°C.

To calculate the fold-change in chickens infected with different strains, the comparative or ΔΔCT method was used. The ΔCT value for each sample (infected tissue) and calibrator (normal tissues) was determined by subtracting the CT value of the RdRp gene and the CT value of the GAPDH gene. Afterwards, the ΔΔCT value for each specimen was determined by subtracting the ΔCT value of the calibrator from the ΔCT value of the sample. The normalized level of the target gene is calculated by the formula:

Normalized RdRp gene level=2^(–ΔΔCT)

Statistical Analysis

Statistical analysis was performed by the Student t-test with multiple comparisons to identify the significant differences. All data are presented as mean ± standard deviation (M ± SD). P-value ≤ 0.05 was considered significant and P-value ≤ 0.01 was considered highly significant.

Results

Identification of Field Strains

Conventional PCR followed by sequencing and sequence analysis confirmed the same variant in the three organs. Virus types A and B were detected in all target organs and virus type C was detected in the trachea and kidney tissues exclusively. The distinct sequences were obtained for A, B, and C variants in comparison with the reference strain (Figure 1).

Figure 1. Sequence homology between RdRp genes of IBV strains detected by PCR and sequencing analysis. IBV reference sequence (AY692454) compared with three (A, B and C) identified variants.

Clinical Findings

No clinical sign was observed in the chickens of the control group. Pathologic lesions were observed in chickens in the groups infected with A, B, and C genotypes. In the chickens of group A (A genotype), respiratory signs, as well as respiratory and kidney gross lesions were found in necropsy. Moreover, respiratory signs along with watery diarrhea and respiratory lesions were revealed in all chickens infected with B genotype. In chickens infected with C genotype, besides the respiratory signs, apparent nephrosis was detected in the kidneys. The mortality rate was 2.78%, 4.12%, and 7.37% in groups A, B, and C, respectively.

Relative Quantification of Virus

The RdRp gene of IBV was amplified and quantified by real-time PCR (Figure 2). Virus type C had the highest quantity (17.02±5.22) and virus type A showed the lowest quantity (5.68±2.4) in infected tissues. Relative viral quantity in all groups was significantly higher than controls. The comparison of virus types demonstrated that the groups infected with types A and C had a highly significant difference. No statistically significant difference was observed between types A and B or B and C.

As shown in Table 1, the relative viral quantity was compared between distinct tissues. The highest viral quantity was detected in the kidney and trachea of the chickens in group C (Figure 3). Furthermore, the lowest quantity of the virus in the lungs was observed in this group. Virus type B showed the highest quantity in the lung and the lowest quantity in the kidney. A low viral quantity was found in all the tissues of chickens infected with virus type A (Figure 3).

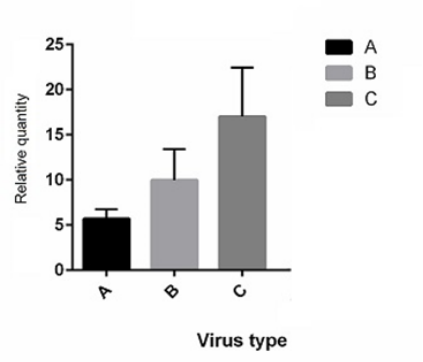

Figure 2. Graph showing the relationship between IBV types (A, B and C) based on the RdRp gene polymorphism and relative viral quantity.

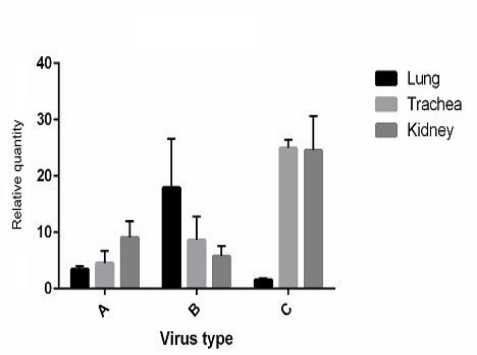

Figure 3. Comparison of relative viral quantity in lung, trachea and kidney between groups of chicken infected with different type of IBV (A, B and C).

Table 1. Values of relative viral quantity in kidney, trachea and lung of chickens infected with three different type of IBV (A, B and C).

Virus	Tissue	Mean	SD
A	Kidney	9.09	3.82
	Trachea	4.52	2.31
	Lung	3.42	1.02
B	Kidney	5.76	1.64
	Trachea	8.61	3.35
	Lung	17.92	7.26
C	Kidney	24.56	6.11
	Trachea	24.94	3.05
	Lung	1.55	0.57

Discussion

Infectious bronchitis in broilers can cause poor weight gain, the condemnation at processing, and mortality due to respiratory disorders and kidney disease. Here, we inspected the association of viral quantity with virus type and mortality in broiler flocks. The IBV exists in diverse genetic forms making detection and disease control very difficult. We conducted real-time reverse transcription PCR for the identification and relative quantification of the virus targeting the RdRp-coding region of the replicase gene of IBV. Among three RdRp genotypes detected in the current study, the viral quantity was 2.99 folds higher in the group infected with virus type C than the group infected with virus type A. Mortality in group C was considerably (7.37%) higher than the other groups, which can show the effects of viral quantity on mortality. In broilers, the strains of some IBV pathotypes cause tracheal lesions and respiratory disease with low mortality. Higher mortality rates have been reported in birds infected with nephropathogenic strains (up to 25%) (Domanska-Blicharz *et al*., 2020; Ignjatović and Sapats, 2000; Kato *et al*., 2019). It is generally accepted that IBV pathogenesis is associated with virus replication rates (Okino *et al*., 2014 & 2017). In the same populations, variations in host-pathogen interactions are correlated with pathogen variation. The associations between IBV strains and pathogenesis in broiler flocks are not fully elucidated.

Increased viral quantity was found in both tracheal and renal samples of chickens infected with type C and lung specimens of group B. These different patterns of viral replication could be related to innate immune responses and virus potentials for replication (Santos *et al*., 2018; Larsen *et al*., 2019). The correlation between the IBV replicase gene and virus virulence has been discussed earlier (Armesto *et al*., 2009). It has also been revealed that the decreased pro-inflammatory response due to the downregulation of TLR7 may be associated with renal lesions (Jang *et al*., 2013 & 2018; Okino *et al*., 2017; Khan *et al*., 2020). However, both immune response dysregulation and/or viral replication behaviors are closely related to virus genes and their roles in pathogenesis.

Conclusion

Polymorphism at the replicase gene of IBV field strains has been revealed to be significantly correlated with viral quantity in the lung, trachea, and kidney. Assays based on the RdRp gene enable the assessment of viral quantity measurement and the obtained data indicate the suitability of this procedure as a tool for epidemiologic and pathogenesis studies. Our findings update the existing knowledge on the phenotypic and genetic relationships and diversity of the new targeted gene of the IBV strains.

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Conflict of Interest

The authors declared no conflict of interest.

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مجله طب دامی ایران، ۱۴۰۰، دوره ۱۶، شماره ۱، ۵۷-۶۳

تشخیص واریانت های ویروس برونشیت عفونی طیور در بافت های نای، ریه و کلیه طیور گوشتی مبتلا با روش افزوده سازی در زمان واقعی

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چکیده

زمینه مطالعه: عامل مهم بروز سویه‌های جدید ویروس برونشیت عفونی طیور، توان بالقوه زیادی است که ویروس برای تغییرات ژنتیکی دارد. حاصل اغلب این تغییرات ژنتیکی، تفاوت‌هایی است که در حدت، تمایل بافتی و میزان تکثیر سویه‌های ویروس در بافت مبتلا مشاهده می‌شوند.

هدف: این تحقیق به‌منظور تشخیص واریانت‌های ویروس برونشیت عفونی طیور در بافت‌های نای، ریه و کلیه طیور مبتلا صورت گرفت. ارتباط احتمالی هر واریانت با میزان نسبی ویروس در بافت‌های نای، ریه و کلیه نیز بررسی شد.

روش کار: واریانت‌ها با روش واکنش زنجیره‌ای پلی‌مراز و متعاقب آن تعیین توالی مشخص شدند. از میان نمونه‌های جمع‌آوری‌شده از گله‌های آلوده در یک فصل (تابستان) از استان‌های گلستان و مازندران، ۹ گله بر اساس واریانت تشخیص داده شده انتخاب شدند به شکلی که برای هر واریانت بتوان از ۳ گله مبتلا نمونه گرفت. از هر گله نمونه‌های بافتی نای، ریه و کلیه از ۵ پرنده آلوده مورد آزمایش قرار گرفت. در ضمن میزان نسبی ویروس در بافت‌های نای، ریه و کلیه برای هر واریانت با روش کمیت‌سنجی نسبی افزوده‌سازی در زمان واقعی تعیین و مقایسه شد.

نتایج: بر اساس نتیج توالی‌یابی سه واریانت ویروس تشخیص داده شد (A, B و C). ویروس‌های A , B در هر سه بافت مورد مطالعه تشخیص داده شد ولی ویروس C در بافت نای تشخیص داده نشد. ویروس C بیشترین میزان (۱۷,۰۲ ± ۵,۲۲) و ویروس A کمترین میزان (۵,۶۸ ± ۲,۴) را در بافت‌های آلوده نشان دادند. میزان نسبی ویروس در بافت پرندگان مبتلا با واریانت ویروس مرتبط بود.

نتیجه‌گیری نهایی: تنوع ژنتیکی سویه‌های ویروس برونشیت عفونی ارتباط معنی‌داری با میزان تکثیر ویروس در بافت‌های نای، ریه و کلیه دارد. یافته‌های این تحقیق گامی است در جهت کشف ارتباط ژنوتیپ ویروس با حدت و بیماری‌زایی.

واژه‌های کلیدی: بافت، جوجه، ، کمیت‌سنجی، گوشتی، ویروس برونشت عفونی

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